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Abstract

The levels and activity of Src non-receptor tyrosine kinases are frequently found to be aberrantly elevated in mammary carcinoma cells compared to non-tumorigenic mammary epithelial cells. However, the role that Src family kinases play in tumor progression remains unclear. The overall objective of this study is to better elucidate the role that endogenous c-Src plays in mammary epithelial tumorigenesis. Our lab has found that genetic and pharmacological inhibition of Src in tumor cells that mimic the early stages of tumorigenesis (T4-2 cells), when cultured in a protein rich extracellular matrix (3D-rBM; Matrigel™), formed organized, polar, multicellular spheroid structures (termed ‘acini) similar to the physiological lobular-aveoli structures found in the mammary tissue. Additionally, more invasive carcinoma cells (MDA-MB-231 cells) whereby Src signaling was pharmacologically or genetically inhibited were unable to form actin-rich invasive structures in 3D-rBM culture. To further characterize the effects that Src inhibition has on tumor cell invasion, we isolated purified pools of MDA-MB-231 expressing EGFP alone or an EGFP tagged dominant negative mutant version of Src (EGFP-dnSrc; K295R, Y527) by flow cytometric cell sorting. Using transwell migration and invasion chamber assays, we found that MDA-MB-231 expressing EGFP-dnSrc displayed decreased migratory and invasive capabilities. EGFP-dnSrc expressing MDA-MB-231 cells also displayed a slower rate of attachment to various cell culture substrates such as plastic, laminin, and Matrigel™. Somewhat surprisingly, we did not detect significant differences in proliferation rates between cells expressing EGFP alone and cells expressing EGFP-dnSrc indicating that Src plays a greater role in migration and invasion than proliferation in MDA-MB-231 cells. Preliminary analysis of members of the Rho family of GTPases also showed that levels of Rho[GTP] and Rac[GTP] but not of CDC42[GTP] were decreased in MDA-MB-231 cells expressing EGFP-dnSrc when incubated in 3D-rBM cultures.

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Introduction

The study of the non-receptor kinase c-Src and its oncogenic variant, v-Src, has provided insight into the role that tyrosine phosphorylation plays in cellular transformation. To date, fibroblasts expressing v-Src have been a main tool used to investigate this question (Meijne et. al. 1997, Moissoglu and Gelman 2003). These studies have shown that v-Src plays a variety of cellular roles that range from cell cycle regulation to the regulation of cell motility and invasion; these processes are deregulated during tumorigenesis. Additionally, the level and/or activity of c-Src have been demonstrated to be elevated in tumors from various tissues such as breast, colon, and prostate (Irby and Yeatman, 2000). However, while c-Src levels/activity is increased in tumors, the role of endogenous Src kinase in carcinoma cells, which are epithelial-derived, remains unclear. Recent studies indicate that the role of c-Src in tumor cells may differ from the role of v-Src in transformed fibroblasts, with c-Src playing a greater role in motility and invasion than in mitogenic transformation (Frame, 2004; Serrels et. al. 2006). Furthermore, there is strong evidence to suggest that tumor cell behavior is context specific and dependent on the three dimensional (3D) architecture of the surrounding microenvironment (Bissell, 2005). Therefore, cell culture and/or mouse model systems that better recapitulate the physioglogical biology of mammary tumors are needed.

With the development of novel cell culture systems composed of reconstituted basement membrane matrices (3D-rBM) that better approximate the microenvironment of mammary tumor cells, recent work has begun to elucidate the role Src family of tyrosine kinases in tumorigenesis. Expression of v-Src in the non-tumorigenic mammary cell line, MCF-10A induces luminal filling and disruption of acinus architecture in 3D-rBM cultures (Reginato, 2005). Additionally, another study showed that expression of the growth factor receptor, CSF-1R, disrupted acinus architecture in 3D-rBM cultures, a process that is dependent on Src activity (Wrobel et. al. 2004). A study examining colon carcinoma cells showed that a Src inhibitor, Dasatinib, did not inhibit cell proliferation in colon cancer cells but did inhibit invasion and migration of colon cancer cells (Serrels et. a. 2006). Carragher et. al. (2006) showed that tumor cells that adopt a more mesenchymal mode of invasion and invasion were particularly sensitive to Src inhibitors and that the invasion was mediated by the extracellular matrix protease Calpain-2.

This proposal aims to examine the effect that inhibition of endogenous Src activity has on mammary carcinoma cells lines. Using the Src-specific pharmacological inhibitors, SU6656 and PP2 we found that inhibition of Src led to formation of acini structures in tumorigenic, non-invasive T4-2 cells when these cells were cultured in 3-dimensional reconstituted basement membrane matrices (3D-rBM; Matrigel). These acini structures were organized, polar multicellular colonies as assessed by the basal relocalization of integrin $\alpha 6$ and the apical orientation of the golgi matrix protein, GM130. Western blot analysis of whole cell lysates and in-vitro kinase assays were used to confirm that Src activity was indeed inhibited at the concentrations of PP2 used. Additionally, inhibition of Src in the more invasive carcinoma line, MDA-MB-231 led to inhibition of invadopodia formation in 3D-rBM cultures. The studies suggested that Src played multiple roles during mammary tumor progression that included disruption of epithelial cell architecture in early tumorigenesis to the acquisition of invasive potential during later stages of tumor formation.

Body

Progress Summary for Award Year April 2006 to April 2007 (As related to 'Statement of Work' in initial grant proposal).

Task 1. To develop additional methods of Src inhibition in mammary epithelial carcinoma cells.

Previous work summary: To validate the findings with the small molecule inhibitors presented in initial proposal and in the followup Summary Report for 4-05 to 5-06, we proposed to focus on the introduction of an EGFP-tagged dominant negative mutant of Src (K297R, Y527F; EGFP-dnSrc) as an additional method to inhibit Src. This mutant form of Src has been used previously to inhibit Src signaling (Gonzalez et. al. 2006). Src inhibition by RNA interference was proposed in the original Statement of Work but our decision to focus on the dominant negative mutant was due, in part to possible functional redundancy between Src family kinase family members Src, Fyn, and Yes. In addition, the potential difficulty of introducing multiple short hairpin RNAs that would concomitantly downregulate the three major family members made expressing dnSrc to be a feasible alternative to RNA interference. We found that the majorityT4-2 cells expressing high levels of EGFP-dnSrc failed to expand and remained single cell units in 3D-rBM cultures suggesting an inhibition of proliferation mediated by Src inhibition. About 1% of cells expressed moderate levels of EGFP-dnSrc and expanded to form acini-like structures as assessed by phase contrast microscopy. However, because of the low percentage of cells expressing EGFP-dnSrc, it was difficult to pursue this line of investigation further. We attempted to establish an inducible system expressing EGFP-dnSrc using the tet-off system. While we were able to obtain clones stably expressing the tetracycline transactivator (TA), we were unable to obtain double selectants that had integrated the EGFP-dnSrc under the control of the tetracycline response element (TRE-EGFP-dnSrc).

We had also shown that pharmacological inhibition led to inhibition of invadopodia in MDA-MB-231 cultured in 3D-rBM suggesting a role of Src decreasing the invasive potential of more aggressive mammary carcinomas. As with the T4-2 cells, we proposed to focus on the expression of EGFP-dnSrc in these cells to validate the pharmacological data. Stable pools of MDA-MB-231 cells expressing of EGFP-dnSrc under the LTR promoter in the pBabe vector displayed a reduced ability to form invadopodia in 3D-rBM confirming the data obtained with the small molecule inhibitors.

Progress of Task 1 From April 2006 to April 2007:

Flow cytometic purification of EGFP and EGFP-dnSrc pool: We were successful in obtaining stable pools of MDA-MB-231 expressing EGFP alone (as an overexpression control) or EGFP-dnSrc under the control of the CMV promoter. Using flow cytometry, we purified populations of MDA-MB-231 cells expressing low or high levels of EGFP ('EGFP-lo' and 'EGFP-hi' respectively) and cells expressing low or high levels of EGFP-dnSrc ('EGFP-dnSrc-lo' and 'EGFP-dnSrc-hi' respectively). Purified populations of MDA-MB-231 were cultured on 3D-rBM for 10 days. Figure 1, Appendix 1 shows represent fluorescent and phase contrast microscopic images of the four purified populations. EGFP expressing cells invaded extensively in 3D-rBM cultures while EGFP-dnSrc-hi populations showed the lowest levels of invasion in 3D-rBM cultures. EGFP-dnSrc-lo populations displayed an intermediate phenotype with moderate invasion

in 3D-rBM cultures. Flow cytometric analysis indicated that following serial passaging, EGFP-dnSrc expressing populations gave rise to low or non-expressing populations even post sorting (data not shown). We decided to focus on low passage EGFP-lo (termed EGFP for rest of report) and EGFP-dnSrc-hi (termed EGFP-dnSrc for rest of report) populations for future characterization and mechanism analysis as these populations expressed comparable levels of EGFP.

Task 2. To monitor the phenotypic effect Src inhibition has on polarity, motility, and invasion in breast carcinoma cell lines.

Previous Work Summary: Using antibodies against polarity markers, we had found that PP2 inhibited T4-2 cells organized into polar multicellular structures (see: Task 1: Previous work summary and Previous Annual Progress Report). Because of the inability to obtain stable cells expressing EGFP-dnSrc or to develop a tet-inducible system with the T4-2 cells, we have proposed to focus on the MDA-MB-231 cells which represent a more amenable system. Previous work indicated that PP2 inhibited MDA-MB-231 cells were less invasive as determined morphologically in 3D-rBM culture assays and less motile as determined from scratch assays.

Progress of Task 2 From April 2006 to April 2007:

Effect of Src inhibition on tumor cell migration and invasion: To confirm our findings with the 3D-rBM cultures and scratch assays, migration/invasion transwell assays were used to quantify the degree of migration and invasion in sorted pools. These migration/invasion chambers are composed of an upper chamber where the cells are seeded and a lower chamber containing a chemoattractant. The chambers are separated by a filter composed of pores of a defined size. Migratory cells cultured in the upper chamber will migrate towards the chemoattractant and migrate through the pores and attach to the bottom side of the filters after a defined period of time. The number of cells can be quantified by analyzing the number of cells that have attached to the underside of the filter with microscopy. To examine the extent of invasion, the filters are coated with a thin layer of extracellular matrix (Matrigel™) in the upper chamber. Invading cells will degrade the Matrigel™ and migrate through the filters. We compared the invasive and migratory capacity of these cells using the 8μM pore sized migration/invasion chambers. Figure 2, Appendix 1 shows that EGFP-lo pools of cells have a high migratory and invasive capacity as compared to cells expressing EGFP-dnSrc-hi pools. Mouse fibroblast NIH 3T3 cells were used as a non-invasive control cell line. While NIH3T3 display high migratory capacity, they were unable to invade through a Matrigel coated transwell chamber. These data confirmed our observations that Src played a role in invasion and migration.

Effect of Src inhibition on tumor cell attachment: To metastasize, tumor cells must initially detach from neighboring cells and local environment and reattach at distant sites. Decreased migration and invasion of EGFP-dnSrc-hi cells may results from inability to form attachments to extracellular matrix substrates. To test this, EGFP and EGFP-dnSrc cells were trypsinized and 1x10e5 cells were seeded on plastic alone or extracellular matrix coated chambers coated with laminin or Matrigel ™. The supernatant of these cultures were collected at 1, 2, 4, 8hours and the number of cells counted to measure the number of cells that had remained unattached. This gives a measure of the degree and kinetics of cell attachment to various substrates. Figure 3, Appendix 1 shows that regardless of substrate, there was a delay of attachment, but not complete inhibition in

EGFP-dnSrc cells compared to EGFP cells indicating that Src plays a partial role in the initial attachment event of cancer cells. This may contribute to the decreased capacity of EGFP-dnSrc expressing cells to invade and migrate. We are currently examining whether formation of various actin-based structures involved in migration and invasion are altered upon Src inhibition when MDA-MB-231 cells are cultured in extracellular matrix substrates.

Effect of Src inhibition on tumor cell proliferation: Src family of kinases have been shown to play a major role in proliferation in fibroblast cells expressing the oncogenic v-Src. Whether Src plays an important role cell proliferation in tumorigenic epithelial cells remains unresolved. Studies have shown that Src inhibition in colon cancer cells (Serrels et. al. 2006) has no effect of cells proliferation. However, other studies have showed that Src inhibition can repress cell proliferation via increased expression of the cell cycle inhibitor p27 (Gonzalez et. al. 2006, Chu, et. al. 2007). In our laboratory, expression of EGFP-dnSrc in T4-2 cells appeared to inhibit cell proliferation as cells stably expressing high levels of EGFP-dnSrc failed to expand and form multicellular acini. To examine whether Src inhibition also inhibited cell proliferation in MDA-MB-231 cells, growth curve analysis of EGFP-lo and EGFP-dnSrc-hi cells were made. Figure 4, Appendix 1 shows a representative growth curve on a semi-logarithmic scale. No significant difference in population doubling times were observed beween EGFP and EGFP-dnSrc pools. Thus, in MDA-MB-231 cells, Src inhibition does not appear to significantly inhibit proliferation.

Effect of Src inhibition on extracellular matrix degradation: We are currently assessing whether inhibition of Src family of kinases prevents the ability of invasive cells to degrade extracellular matrix substrates by analyzing the degradation of FITC-gelatin coated on coverslips.

Task 3. To examine the mechanism by which Src inhibition exerts its phenotypic effects on breast carcinoma cell lines.

Previous Work Summary. It was previously shown that PP2 mediated Src inhibition of T4-2 cells led to a dose-dependent decrease in AKT phosphorylation at serine 473 and of ERK phosphorylation. Downregulation of integrin β1 or epidermal growth factor, (EGF-R) was not observed in Src inhibited T4-2 cells despite the findings by other studies that showed a concomitant decrease in EGF-R and integrin β1 upon acinus formation by various signaling inhibitors. We concluded that inhibition of EGFR and integrin β1 was sufficient but not necessary for acinus formation in T4-2.

Progress of Task 3 From April 2006 to April 2007:

Effect of myristoylated AKT1 on acinus formation in T4-2 3D-rBM cultures: To determine whether AKT1 signaling was necessary for the disruption of acinus formation in T4-2 cells, an AKT mutant containing a myristoylation signal (Myr-AKT1; constitutively active) was stable expressed in T4-2 cells. Our reasoning was that if AKT signaling was necessary for the disruption of acinus formation in 3D-rBM cultures, a constitutively active mutant should rescue the effect of Src inhibition. Figure 5, appendix 1 shows phase contrast images of T4-2 cells cultured in 3D-rBM matrices expressing myr-AKT1 in the presence or absence of PP2. Cells expressing myr-AKT1 formed larger and disorganized colonies at PP2 concentrations that induced acinus formation in PP2. These data support the finding that downregulation of AKT1 by Src inhibition was required for acinus formation in 3D-rBM cultures.

Regulation of Rho family of GTPases in Src inhibited MDA-MB-231 cells: To examine the mechanism by which Src inhibition prevents invadopodia formation, we focused on the activation of Rho family GTPases. Studies have shown that tumor cells can adopt predominant an 'ameboid' or a 'mesenchymal' phenotypic form of migration when cultured in extracellular matrices. In ameboid migration, tumor cells form membrane 'blebs' through the extracellular matrix. This mode of migration does not require degradation of extracellular matrix and appears to be Rho dependent. In mesenchymal migration, cells form extended actin rich invadapodia structures to degrade the extracellular matrix; this mode is Rac and CDC42 dependent. MDA-MB-231 cells adopt a mesenchymal-like form of migration in Matrigel™. However, cells expressing EGFP-dnSrc fail to form actin protrusions and appear to adopt a more ameboid mode of migration. We postulated that Src was inhibiting Rho activation and stimulating Rac or CDC42 activation. Therefore, Src inhibition would lead to increased levels of Rho[GTP] and decreased levels of Rac[GTP] or CDC42[GTP]. We used GST-Rhotekin or GST-PAK21 as affinity reagents to precipitate Rho[GTP] and Rac/CDC42[GTP] respectively from whole cells lysates. Although the data need to be validated, preliminary results indicated that both Rho[GTP] (Figure 6, Appendix 1) and Rac[GTP] (Figure 8, Appendix 1) but not CDC42[GTP] (Figure 7, Appendix 1) levels were decreased in EGFP-dnSrc expressing MDA-MB-231 cells compared to EGFP expressing cells. Since the major extracellular component of Matrigel™ is the glycoprotein, laminin, we cultured MDA-MB-231 cells on laminin and examined levels of Rho[GTP] levels. Similar to cells cultured on Matrigel™, MDA-MB-231 cells expressing EGFP-dnSrc cultured in laminin-coated wells show decreased Rho[GTP] levels compared to MDA-MB-231 cells expressing EGFP alone (Figure 6, Appendix 1). Treatment of MDA-MB-231 cells with PP2 also decreased Rho[GTP] levels (data not shown). If decrease of Rho[GTP] and Rac[GTP] is confirmed, we will subsequently examine whether downregulation of Rho or Rac activation is necessary for the inhibiton of invadopodia. This will be done by introducing activated mutants of RhoA (Q63L) and Rac1 (Q61L) into cells expressing EGFP-dnSrc and analyzing whether the mutants can rescue the phenotypes observed with EGFP-dnSrc. Conversely, we are introducing dominant negative mutants of RhoA and Rac into MDA-MB-231 cells to examine whether inhibition of Rho or Rac signaling is sufficient to prevent invadopodia formation. In addition, we are currently investigating whether upstream regulators of Rho family of kinases are differentially regulated between cells expressing EGFP compared to cells compared EGFP-dnSrc. To that end, we are currently assessing the phosphorylation status of the guanine dissociation inhibitor, RhoGDI, since it has been reported that phosphorylation of RhoGDI by Src can lead to decreased interaction with Rho and Rac (DeMardirossian et. al. 2006) and decrease the inhibitory action of RhoGDI. We therefore postulate that inhibition of Src leads to decreased RhoGDI phosphorylation and increases the interaction and inhibitory action of RhoGDI. We also plan to examine the activation levels of the stimulatory upstream mediators, RhoGEFs and the inhibitory RhoGAPs to determine the mechanism by which Src inhibition leads to downregulation of Rho and Rac activation.

Key Research Accomplishments and Conclusions:

- Established purified populations of MDA-MB-231 cells expressing EGFP or EGFP dnSrc by flow cytometric cell sorting.
- Showed that sorted populations of MDA-MB-231 cells expressing EGFP-dnSrc displayed a decreased invasive phenotype in 3D-rBM cultures confirming the pharmacological data.
- Showed that MDA-MB-231 cells expressing EGFP-dnSrc displayed decreased migration and invasive properties as well delayed attachment to various extracellular matrix substrates. Cell proliferation was not significantly altered between MDA-MB-231 expressing EGFP and MDA-MB-231 cells expressing EGFP-dnSrc. Thus, Src kinase activity in MDA-MB-231 appears to play a greater role in migration and invasion than cell proliferation.
- Showed that expression of mysritoylated-AKT1 can inhibit acinus formation induced by Src inhibition in T4-2 cells. The data are consistent with previous findings that AKT phosphorylation are decreased in Src-inhibited T4-2 cells. This suggests an important role of AKT signaling in the disruption of acinus formation.
- Preliminary results indicate that Rho[GTP] and Rac[GTP] but not CDC42[GTP] levels are decreased in MDA-MB-231 cells expressing EGFP-dnSrc. Thus Rho and Rac may represent important downstream effectors of Src signaling in mediating the invasive phenotype of MDA-MB-231 cells.

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Appendix 1: Supporting data and figures

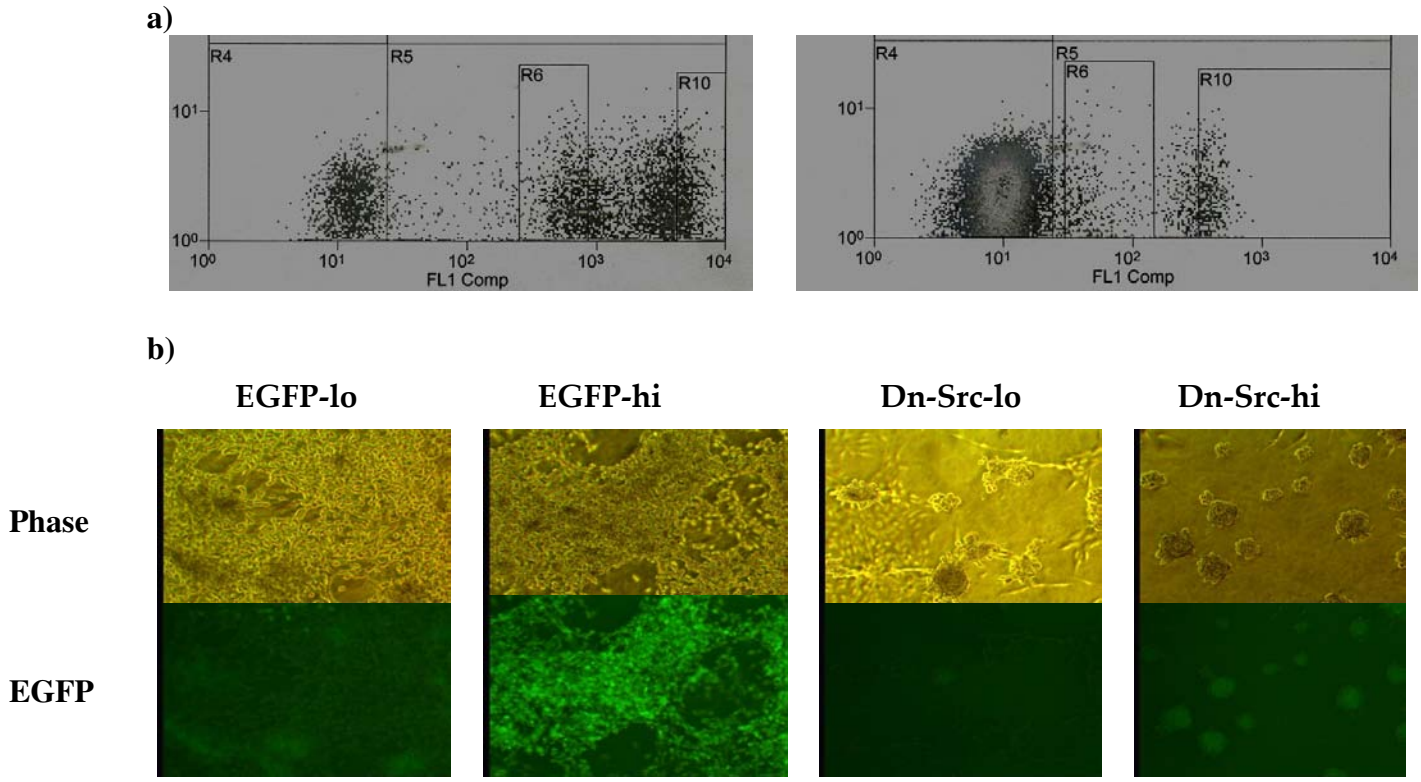

Figure 1: a) FACS profile of stable pools of MDA-MB-231 cells. Cells were transfected with pLEGFP or pRev-tre-EGFP-dnSrc and incubated in selection media (DMEM+10%FBS+1mg/ml G418 for pLEGFP or 1mg/ml hygromycin for pRev-tre-EGFP-dnSrc) for three weeks. Cells were harvested and resuspended at a concentration of 1x10e7 cells/ml and sorted using a DAKO cytomation MOFLO high speed cell sorter equipped with an argon-ion gas laser for sorting EGFP positive cells. The left panel shows the flow profile of the EGFP expressing pool while the right panel shows the EGFP-dnSrc expressing pool. Based on the profile, populations were gated for "high expressors" and "low expressors." Gate R6 and R10 population in left panel were classified as EGFP-lo and EGFP-hi respectively. Gate R6 and R10 population in right panel were classified as EGFP-dnSrc-lo and EGFP-dnSrc-hi respectively. **b) Phenotype of sorted populations in 3D-rBM cultures.** Representative phase contrast and fluorescent images of sorted populations from a) in 3D-rBM cultures are shown. Sorted populations were seeded on a 500µL Matrigel ™ layer in a 6-well chamber (at 1x10e5 cells per chamber) with an overlay of complete growth media containing 5% Matrigel™ and incubated for 10days before fluorescent and phase contrast microscopy analysis.

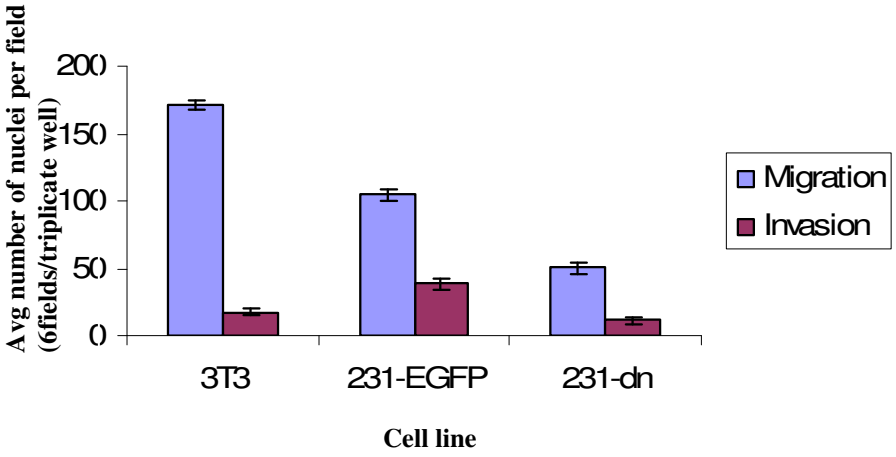

Figure 2: Migration and invasion profile of sorted pools. For analysis of migration, 1x10e5 cells of each line were trypsinized and seeded in the upper chamber of an 8µM pore, non-Matrigel™ coated transwell containing DMEM with 0.1% BSA. DMEM with 10% FBS was added to the lower chamber of the transwell. For analysis of invasion, cells were seeded in the upper chamber of a Matrigel-coated transwell. DMEM with 10%FBS was added to lower chamber as with the migration chamber. NIH3T3 cells were used a non-invasive control. Cells were incubated for 24hours and the transwell filters were fixed, permeabilized and stained with DAPI to visualize the nuclei of cells that had migrated/invaded to the underside of the filter. Six fields from triplicate wells were counted. Graph shows average number of nuclei counted per field +/- S.E.M.

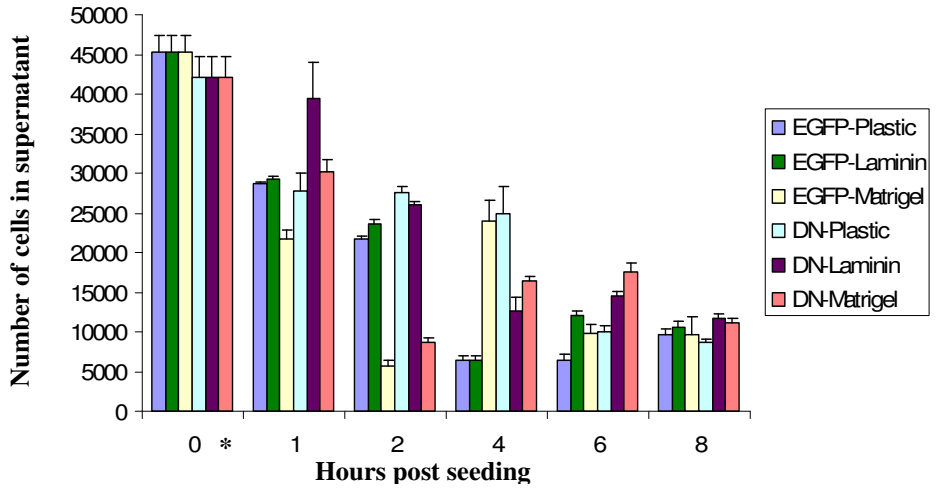

Figure 3: Cell attachment of EGFP and EGFP-dnSrc expressing MDA-MB-231 cells on extracllular matrices. Cells were trypsinized, washed 1x with DMEM+10% FBS and seeded (at 5x10e4 cells per chamber in a 24 well plate) on either plastic, laminin coated, or Matrigel coated substrates. At indicated times, the supernatant of each chamber was collected and the cells were counted in a Beckman coulter counter. Bars represent average number of cells in supernatant from triplicate well +/- S.E.M. Bars at Time=0h (marked with*) represent the average number of cells in supernatant of cells cultured on plastic substrates only for each corresponding pool (EGFP or EGFP-

dnSrc) and were set at equal to each other. Bars at 1, 2, 4, 6, 8h represent number of cells in supernatants of cells cultured on corresponding substrates indicated by the figure legend.

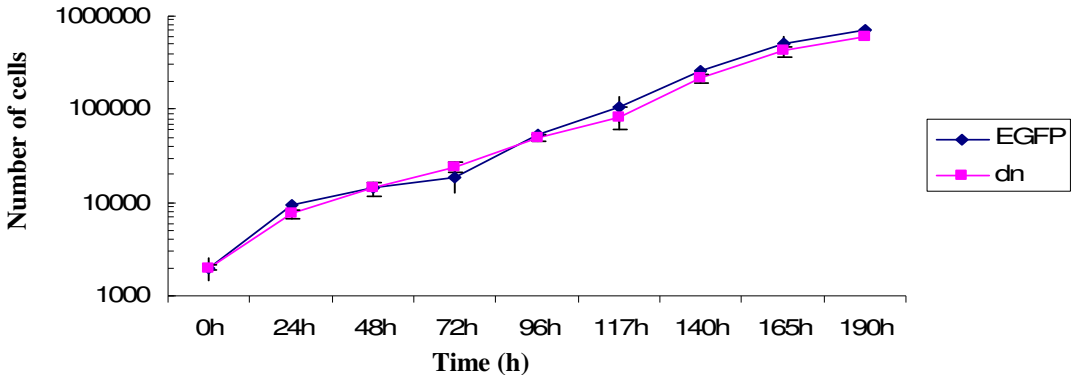

Figure 4: Growth Curve analysis of MDA-MB-231 sorted pools. Cells were trypsinized and subsequently seeded in 24-well cell culture chamber (at 2x10e3 cells per well). At the indicated times, cells were harvested and counted using a Beckman Coulter Counter. Using best line fit analysis, we obtained a linear equation for each growth plot according to the line equation y=mx+b. Average doubling times from three independent experiments for EGFP and EGFP-dnSrc expressing cells was calculated from the derived linear equation. The average doubling time for MDA-MB-231 cells expressing EGFP was calculated to be 20.6+/-1.8 hours while the average doubling time for MDA-MB-231 cells expressing EGFP-dnSrc was calculated to 22.4+/-1.8 hours.

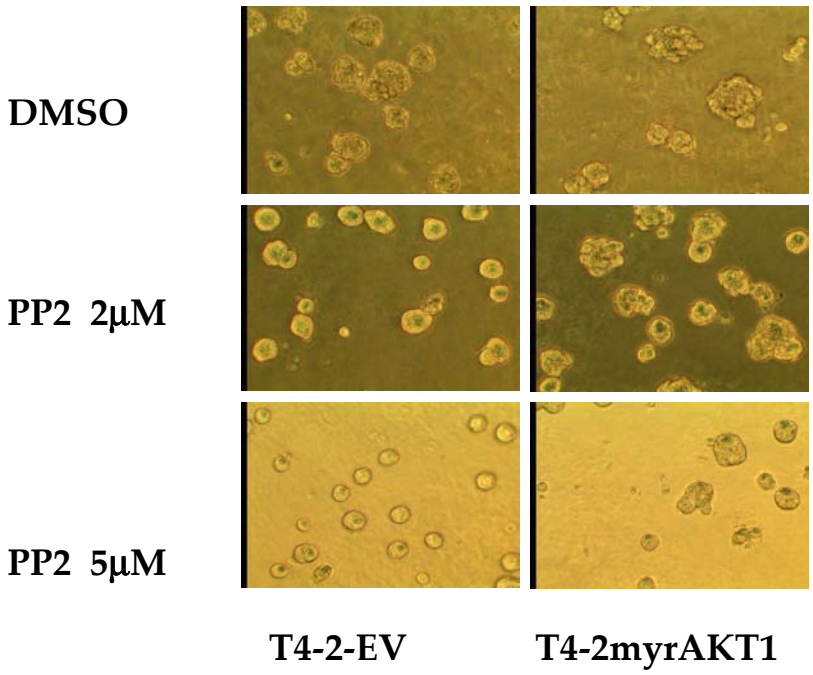

Figure 5: Expression of mysritoylated AKT1 disrupts acinus formation induced by Src inhibition. T4-2 cells were transfected with pLNCX2 alone (EV) or pLNCX2-myr-AKT1 (T4-2myrAKT1) and stable pools were established after three weeks in selection media containing 1mg/ml G414. Stable pools were subsequently cultured in 3D-rBM cultures for 10 days in the presence or absence of 2 or 5μM PP2. Panels shows representative phase contrast images of 3D-rBM cultures.

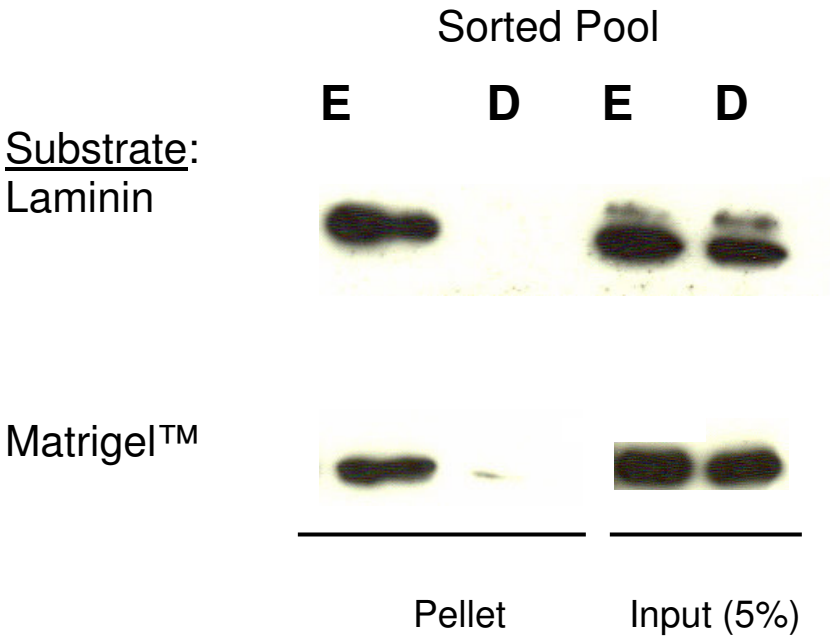

Figure 6: Downregulation of RhoA[GTP] in MDA-MB-231 expressing EGFP-dnSrc. MDA-MB-231 cells expressing EGFP (E) or EGFP-dnSrc (D) were cultured in laminin coated or Matrigel coated 3D rBM cultures for 24hours and subsequently lysed with magnesium containing HEPES

lysis buffer (MLB buffer) containing protease inhibitors. 100µg of GST-Rhotekin-agarose was used to precipitate RhoA[GTP] from lysates ('pellet'). Beads were washed 3x with MLB buffer with protease inhibitors prior to boiling in sample loading buffer and SDS PAGE separation on a 12% polyacrylamide protein gel. Proteins were transferred to a PVDF membrane and the membrane was probed with an anti-RhoA antibody (1:200) followed by incubation with anti-mouse-HRP and subsequent chemiluminiscent detection. Prior to affinity precipitation, 5% (v/v) of each cell lysate was run in parallel to ensure equal amounts of total RhoA proteins in each lysates ('Input').

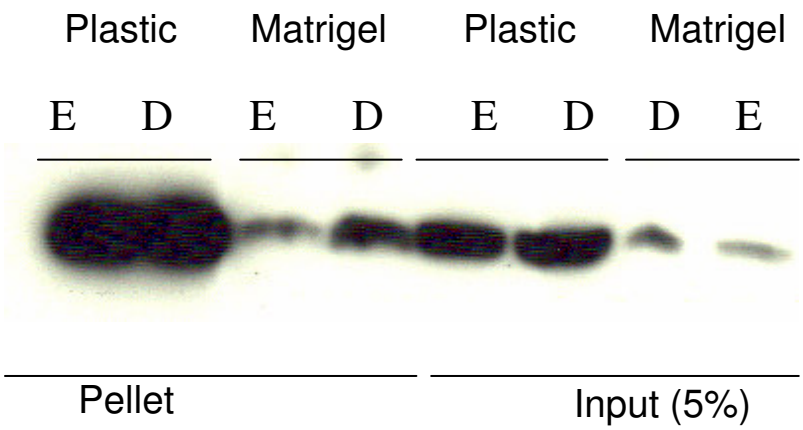

Figure 7: Effect of EGFP-dn on CDC42[GTP] levels. MDA-MB-231 cells expressing EGFP (E) or EGFP-dnSrc (D) were cultured on plastic or in Matrigel coated 3D rBM cultures for 24hours and subsequently lysed with magnesium containing HEPES lysis buffer (MLB buffer) containing protease inhibitors. 20µg of GST-p21 Pak binding domain-agarose was used to precipitate CDC-42[GTP] from lysates ('pellet'). Beads were washed 3x with MLB buffer with protease inhibitors prior to boiling in sample loading buffer and SDS PAGE separation on a 12% polyacrylamide protein gel. Proteins were transferred to a PVDF membrane and the membrane was probed with an anti-cdc42 antibody (1:200) followed by incubation with anti-mouse-HRP and subsequent chemiluminiscent detection. Prior to affinity precipitation, 5% (v/v) of each cell lysate was run in parallel to ensure equal amounts of total CDC42 proteins in each lysates ('Input').

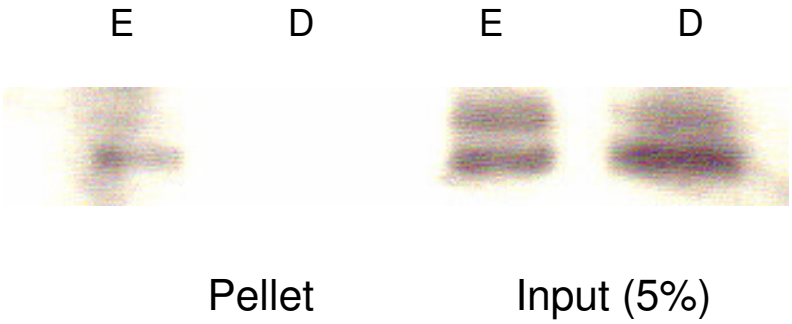

Figure 8: Effect of EGFP-dn on Rac[GTP] levels. MDA-MB-231 cells expressing EGFP (E) or

EGFP-dnSrc (D) were cultured on plastic or in Matrigel coated 3D rBM cultures for 24hours and subsequently lysed with magnesium containing HEPES lysis buffer (MLB buffer) containing protease inhibitors. 20μg of GST-p21 Pak binding domain-agarose was used to precipitate Rac1[GTP] from lysates ('pellet'). Beads were washed 3x with MLB buffer with protease inhibitors prior to boiling in sample loading buffer and SDS PAGE separation on a 12% polyacrylamide protein gel. Proteins were transferred to a PVDF membrane and the membrane was probed with an anti-Rac antibody (1:1000) followed by incubation with anti-mouse-HRP and subsequent chemiluminiscent detection. Prior to affinity precipitation, 5% (v/v) of each cell lysate was run in parallel to ensure equal amounts of total CDC42 proteins in each lysates ('Input').